EXPLOITING IMMOBILIZED ENZYMES; DETOXIFICATION OF NERVE AGENTS

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Abstract

We have developed an alternative means of protecting and decontaminating individuals from exposure to OP nerve agents. We describe here the development of a product, composed of cholinesterases (ChEs), oxime, and polyurethane foam (PUF) combinations, for the removal and decontamination of OP compounds from biological surfaces such as skin. FBS-AChE and equine-BChE were immobilized by covalently linking the enzymes to polyurethane foams having the consistency of sponges. The enzymes attach to the inert foam at multiple points dependent upon the available free aliphatic amines (primarily lysines) on their surface. Based on molecular modeling, the majority of the lysines residues were found on the back side of the ChEs, although a few are close to the rim of the catalytic-site gorge. We found that the PUF had a significantly higher loading capacity for ChEs than the amount of purified ChE added in these studies, implying that even higher potency sponges could be synthesized. The immobilized ChEs showed little leakage from the PUF matrix; after more than 20 wash and assay cycles over three days the sponge retained original activity. The ChE-PUFs showed enhanced thermal stability at elevated temperatures, having 50% activity after 16 h at 80 °C. The ChE-PUFs showed no decrease in activity after more than three years at 4 °C or seven months at 25 °C. OPs such as DFP or MEPQ inhibited the activity of ChE-PUF as was observed for non-immobilized ChE in solution. However, in the presence of the oxime HI-6, the activity of AChE-sponge was not completely inhibited by MEPQ until the molar concentration of OP reached up to 1000 times that of the active site. Equally important, rinsing the sponge with HI-6 in the absence of OP restored nearly all the original cholinesterase activity, permitting the AChE-sponge to be cycled many times. We have demonstrated the rapid copolymerization of ChEs at room temperature; the ChE-sponges exhibit high activity and stability, making them suitable for a wide variety of decontamination tasks.

1. Introduction

Organophosphorus compounds (OPs) are inhibitors of acetylcholinesterase (AChE, EC 3.1.1.7) and butyrylcholinesterase (BChE, EC 3.1.1.8) because they bind specifically to the active-center serine, yielding an inactivated phosphorylated enzyme. The sequel of OP toxicity is a cholinergic crisis. The serine-phosphate bond can either spontaneously reactivate regenerating the active enzyme, or with nerve agents such as soman, the enzyme-OP complex can "age" rapidly, producing an enzyme that is refractory to reactivation by oxime nucleophiles. We have demonstrated an antidotal therapy using cholinesterases to counter the toxicity caused by OP compounds (Maxwell et al., 1992; Caranto et al., 1994; Saxena et al., 1997). The enzyme bioscavenger approach is effective against a variety of OP compounds; pretreatment of rhesus monkeys with fetal bovine serum (FBS) AChE or equine serum BChE protected them against a challenge of up to 5 LD_{50} of soman. While the use of cholinesterase (ChE) as a single pretreatment drug for OP toxicity provided complete protection, a stoichiometric amount of enzyme was required to neutralize the OP *in vivo*. To increase the OP/enzyme stoichiometry, enzyme pretreatment was combined with the oxime HI-6 so that the catalytic activity of OP-inhibited AChE is rapidly and continuously restored before irreversible aging of the enzyme-OP complex can occur. Thus, the OP is continuously detoxified.

In addition to the *in vivo* antidotal therapy, the *in vitro* reactivation of OP-inhibited ChEs by oximes has important applications for medical, surgical, and skin decontamination, and for the decontamination of materials, equipment, and the environment. This is important since currently accepted methods for decontamination of personnel, large areas, and materials use sodium hydroxide and bleach, which are caustic and harmful and also pose a significant environmental burden. With the constant threat of chemical warfare, the development of alternative means of protecting and decontaminating individuals from exposure to OP nerve agents is critical. We describe here the development of a sponge product, composed of ChE, oxime, and polyurethane foam combinations for the removal and decontamination of OP compounds from biological surfaces such as skin.

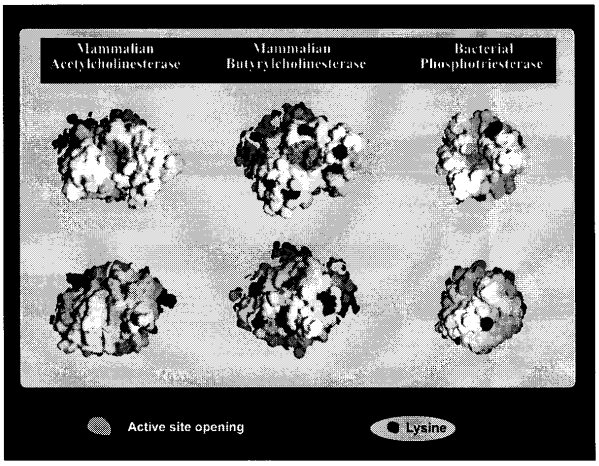


Figure 1

Modeled surfaces of ChEs and triesterase. The top row shows a view of the front of the enzymes with the lip of the active site gorge shaded in the center. The bottom row shows the back side of the enzymes (180° rotation). The Lys residues on the surface, which are potential coupling sites to the polymer, are shaded dark in both the top and bottom row.

It has been previously demonstrated that a variety of enzymes exhibited enhanced mechanical and chemical stability when immobilized on a solid support, producing a biocatalyst. The study of degradation of organophosphates by immobilized enzymes dates back to Munnecke (1979), who attempted to immobilize a pesticide detoxification extract from bacteria by absorption on glass beads. The absorbed extract retained activity for a full day. Wood and coworkers (1982), using isocyanate-based polyurethane foams (Hypol), found that a number of enzymes unrelated to OP hydrolysis could be covalently bound to this polymer and that every enzyme retained activity to varying degrees; after that Havens and Rase (1993) used parathion hydrolase. Based on the observation that polyurethane foams are excellent adsorption materials for OPs such as pesticide vapors (Turner & Glotfelty, 1977), and from our previous studies that soluble ChEs and oxime together have the ability to detoxify OP compounds, we combined these features in a porous polyurethane foam formed *in situ* from water-miscible hydrophilic urethane prepolymers and the enzymes. Thus, we envisioned a reusable OP sponge for decontamination.

2. Methods

2.1 Enzymes: AChE was purified to homogeneity from fetal bovine serum using procainamide-Sepharose 4B affinity chromatography (De La Hoz et al., 1986). Equine serum BChE (Sigma Chemical Co., St. Louis, MO.) was purified similarly.

2.2 Polyurethane foam (PUF or Sponge) synthesis: Hypol prepolymer TDI 3000 was purchased from Hampshire Chemical, Lexington, MA and Pluronic P-65 surfactant from BASF Specialty Chemicals, Parsippany, NJ. A typical synthesis of the sponge consists of enzyme in phosphate buffer containing 1% (final concentration) surfactant and 6 g of prepolymer. The 2-phase system was mixed at 2500 rpm for 50 sec (LeJeune et al., 1997) and injected with a syringe into a suitable mold, such as a 50 mL polypropylene tube or Tupperware® container. The sponge containing the covalently coupled ChEs cured in less than 30 minutes, molded to the shape of its container.

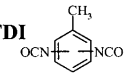
2.3 Determination of enzyme activity: The sponges were evaluated for activity using a modified Ellman method in an aqueous environment of 50 mM phosphate buffer, pH 8.0, containing acetylthiocholine for AChE or butyrylthiocholine for BChE (De La Hoz et al., 1986). The reactions were monitored spectrophotometrically at 412nm in a temperature controlled cuvette containing a stir bar and a small portion of sponge to be assayed, which remained below the light path. The system is two-phases, substrate and coupling to color development of the Ellman assay in the aqueous phase and the ChE-immobilized sponge in the solid phase. We found that there was no adsorption of the final reaction product of the Ellman assay on sponge lacking ChE (no

added protein) or sponge synthesized with bovine serum albumin in place of ChE. Additionally, product generated was linear with time, indicating release of the reaction product to the aqueous environment was not rate limiting.

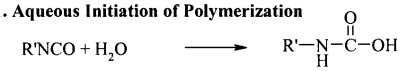
2.4 Inhibition of ChE-PUF by OPs and reactivation with oxime: ChE-PUF were incubated with increasing concen-trations of OP with or without 2 mM oxime reactivator, and the remaining ChE activity in the sponge was determined after rinsing the sponges in phosphate buffer to remove unreacted OP and oxime. To test for reactivation of OP-inhibited ChE-PUF, oxime was added to washed and inhibited PUFs.

2.5 Molecular Modeling of Enzymes: Modeling of the enzymes was performed on a Silicon Graphics workstation using molecular modeling software (Insight II, Biosym Technologies, San Diego, CA).

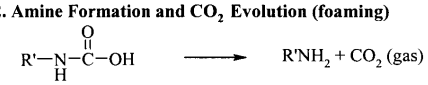
Scheme for ChEs Crosslinked to TDI Polyurethane Foam



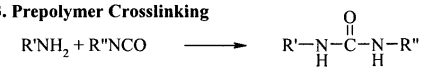
1. Aqueous Initiation of Polymerization



2. Amine Formation and CO_2 Evolution (foaming)



3. Prepolymer Crosslinking



4. Covalent ChE Incorporation at Aliphatic Amino Group(s)

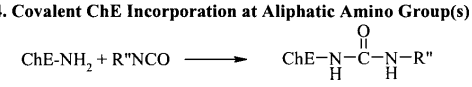


Figure 2

3. Results and Discussion

3.1 Polyurethane foam synthesis. The polyurethane foam comes in a variety of formulations; we describe here the polyether prepolymer derived from tolyl diisocyanate (TDI). The multi-branched polyethylene oxide TDI reacts most favorably with free aliphatic amines such as lysine present on the surface of the ChEs (or any protein) to become a permanent cross-linked part of the elastomeric matrix. Figure 1 shows the lysine residues on the surface of ChEs available for coupling to the prepolymer. Based on molecular modeling, there are at least one Lys and 29 Arg water-accessible residues on the surface of FBS-AChE to couple to the PUF, while 26 Lys and 26 Arg residues were modeled for equine-BChE. The majority of the Lys and Arg residues were found on the back side of the ChEs, and only a few are found on the side of the enzyme where the catalytic site gorge is located. The rim and the catalytic site gorge opening of both AChE and BChE (shaded in figure 1, top row) appeared to be essentially devoid of Lys and Arg. Therefore, coupling these enzymes to PUF should have minimal effect on the entrance of substrate, inhibitors, or reactivators (oximes), release of products of catalysis to and from the active site, and the kinetic rates of the enzymes. The specific reaction of enzyme with polymer proceeds as shown in figure 2. Synthesis is initiated when H_2O molecules react with the isocyanate groups present within the polyurethane prepolymer. Isocyanate reacts with the water to form an unstable carbonic acid, which degrades to an amine yielding CO_2. The CO_2 causes the polymer to rise and become a foam, and simultaneously the amines readily react with the isocyanate groups leading to urea linkages. Since the ChE contains amines that are on the surface and available to react with the isocyanate groups, they can become an integral part of the PUF during

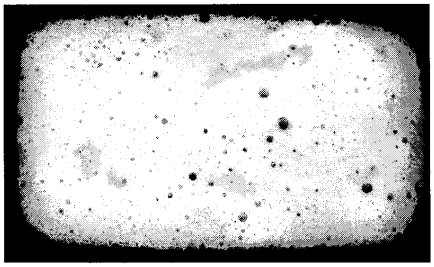


Figure 3
Final product: FBS-AChE-sponge

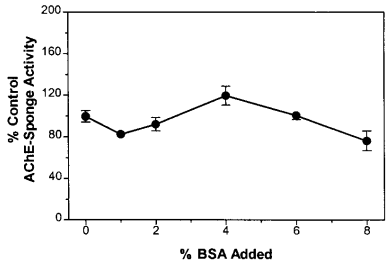


Figure 4
Increasing amounts of BSA were added during synthesis to a constant amount of AChE and TDI polymer.

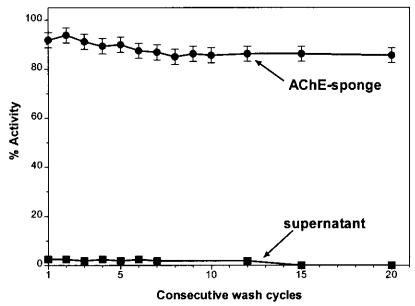


Figure 5

An AChE-sponge was alternately washed with phosphate buffer and assayed for activity. This procedure was carried out for three days. Similar results were observed for BChE-sponge.

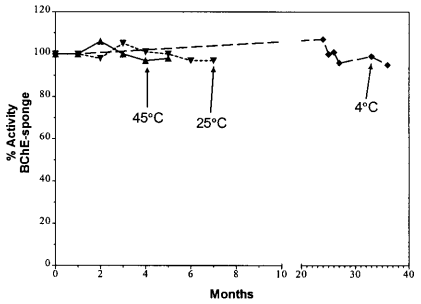


Figure 6

Stability of BChE-sponge at various temperatures. Similar results were obtained with AChE-sponge.

synthesis. There is no significant entrapment of the enzyme in the foam as found with cyclodextrins, or physical adsorption of the enzymes, as observed with activated carbon. The inclusion of a surfactant such as Pluronic P-65 at about 1% final concentration controls the sponges' final structure and wetability. Remarkably, after only vigorous mixing at ambient temperature, the sponge cures in less than 30 minutes, molded to the shape of its container, as shown in figure 3. Therefore, the ChE-sponges could be formed into towelettes to decontaminate skin or 4x4 inch surgical pads to prevent cross-contamination of medical personnel, or even mops to decontaminate sensitive equipment.

3.2 Capacity of ChEs-PUF: Our results demonstrate the following characteristics of sponges containing either immobilized AChE or BChE. After curing, TDI sponges appeared as medium to small open celled structures; the TDI sponge was soft and would be suitable for bathing OP contaminated skin. As expected for a uniform immobilization of AChE or BChE throughout the sponge, a linear correlation was established between the weight of the TDI sponge and enzyme activity. Sponges could be washed with either 50 mM phosphate buffer, distilled water, or 10 mM ammonium bicarbonate without affecting substrate hydrolysis. Different batches of TDI sponges retained about 50% of the original ChE activity. This demonstrates that this simple procedure of *in situ* mixing at 22°C of TDI prepolymer, surfactant, and enzyme yields a useful and effective product retaining much of the original ChE activity, while gaining stability. We found that the TDI sponge has a significantly higher loading capacity for ChEs than the amount of purified AChE we added. When increasing amounts of nonspecific protein (bovine serum albumin, BSA) were added to a constant amount of purified AChE and the mixture cured, there was no reduction in sponge ChE activity, even when there was a 1000-fold excess of BSA, as shown in figure 4. These results suggest that even higher potency sponges can be synthesized from purified ChEs or by synthesizing with additional or other ChEs, thereby substantially increasing the effectiveness of the preparation.

3.3 Stability: When AChE or BChE TDI PUF was exhaustively washed and activity determined, and the wash and assay cycle repeated more than twenty times over three days, no decrease in activity occurred (figure 5), indicating that the sponges could be used repeatedly. These results also demonstrate that the ChEs were covalently cross-linked in the sponge matrix and that the ChEs would not leach out to skin or equipment. While long-term temperature and stability of the ChE TDI sponges are still under evaluation, we have found that ChE activity remains unchanged at 4°C after more than three years and at 25°C after about seven months, or at 45°C after 5 months (the length of the current tests, figure 6). The TDI sponge imparts remarkable stability to the ChE-sponges; about 50% of the original AChE-sponge activity and 20% of the BChE-sponge activity remained after 16 hours at 80°C,

Table 1. Time-Dependent Inhibition of ChEs by MEPQ

ChE	Enzyme Form	Bimolecular rate constant (M^{-1} min^{-1}) ± SD
AChE	soluble	$1.59 \pm 0.52 \times 10^8$
	sponge	$1.00 \pm 0.28 \times 10^8$
BChE	soluble	$4.15 \pm 0.78 \times 10^7$
	sponge	$4.21 \pm 2.00 \times 10^7$

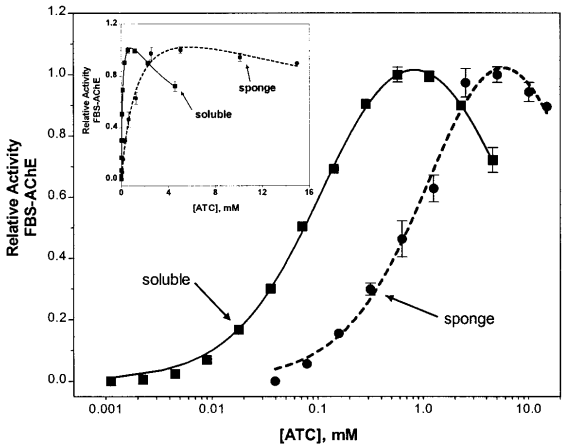


Figure 7

Comparison of soluble AChE and AChE-sponge.

exhibit no activity. The ChE-sponges can be exhaustively dried under vacuum at 22 °C and then rehydrated without loss of the enzyme activity.

3.4 Comparison of ChEs-PUF and soluble ChEs: An initial rates method (using Ellman's assay) was used to determine the parameters K_m, k_{cat}, and k_{cat}/K_m for immobilized and soluble AChE and BChE. The number of active sites of either the coupled or soluble ChEs was determined by titration with MEPQ (7-(methylethoxyphosphinyloxy)-1-methylquinolinium iodide). As shown in figure 7, the K_m values for the AChE-PUF was about 10-fold greater than for the corresponding soluble enzyme (rightward shift of the curve), while k_{cat} values were less dramatically affected. The combined effects on affinity for substrate and k_{cat} resulted in approximately a 20- to 50-fold decrease in acylation (k_{cat}/K_m). A similar rightward shift in the curve was observed for BChE-PUF and soluble BChE. Interestingly, while soluble BChE lacked substrate inhibition, BChE-sponge yielded substrate inhibition. These results suggested that covalent binding of surface residues of ChEs to the polymer caused changes to be transmitted to the active site region of the bound enzymes. In contrast to the change in K_m values for the immobilized ChEs, there was no observed shift in the pH profile of the enzymes, as shown in figure 8. More important, the bimolecular rate constants for the inhibition of AChE-PUF and BChE-PUF and the corre-sponding soluble enzymes by MEPQ at 25 °C, reported in Table 1, showed no significant difference between soluble and covalently bound enzymes. Thus, the OP interacts similarly with soluble and immobilized ChEs.

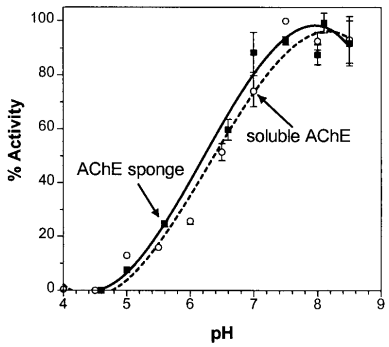


Figure 8

pH profile of soluble AChE and AChE-sponge.

conditions under which soluble enzyme would

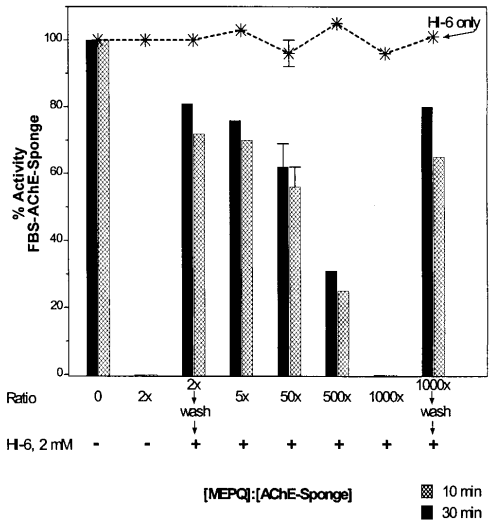


Figure 9

AChE-sponge was treated with OP MEPQ at the indicated molar ratios of MEPQ to enzyme active sites. Note that even after addition of 500-fold molar excess of OP, some enzyme was uninhibited in the presence of HI-6, and that after almost complete inhibition (1000x), the sponge could be rinsed with HI-6 and its activity restored for subsequent use.

3.5 OP inhibition and oxime reactivation of AChE-PUF: Next, we evaluated the ChE-sponges for inhibition by the OPs diisopropylfluorophosphate (DFP) and MEPQ and reactivation by oximes. The activity of AChE-sponge was inhibited by DFP and MEPQ proportionally to the stoichiometric amount of OP added, as observed for non-immobilized AChE in solution. In the presence of 2 mM HI-6, the activity of AChE-sponge was almost completely inhibited by MEPQ only at an [OP]:[AChE-sponge] of 1000 (ratio of the concentration of OP to active sites), as shown in figure 9. However, simply rinsing the sponge with HI-6 in the absence of OP restored most of the original cholinesterase activity, permitting the AChE-sponge to be cycled many times, and the polyurethane matrix retained its sponge-like characteristics during the cycling. Similarly, DFP-inhibited BChE-sponge could be reactivated by TMB4, the more effective oxime for BChE reactivation. Effect of higher doses of more potent OPs such as soman and sarin are being evaluated on the ChE-sponges.

4. Conclusions

4.1: We have demonstrated the rapid *in situ* copolymerization of ChEs and PUF at room temperature, and showed that the ChE-sponges exhibit high enzyme activity, making them suitable for a wide variety of decontamination processes. These TDI ChE-sponges exhibit remarkable long-term stability and increased resistance to elevated temperatures. The immobilized enzymes show similar activity to and the unique properties of their soluble but unbound form.

4.2: The effectiveness of ChE-immobilized sponges for detoxification of OPs is amplified by oxime reactivators. This allows the repeated use of the enzyme-sponges. Since OPs in a diluted aqueous media are more readily absorbed by the skin, the use of the sponge for skin decontamination is the preferred mode: the PUF absorbs the OPs, preventing further penetration through the skin, and the immobilized ChEs detoxify the OPs. In addition, immobilized enzyme-sponges will not cause any immune reactivity when used on wounds or skin, since these enzymes do not leach out as observed in encapsulated preparations such as liposomes or cyclodextrans.

4.3: Due to the large capacity of the prepolymer for protein, high activity sponges can be synthesized from purified ChEs, substantially increasing their efficacy. Multiple OP-hydrolyzing enzymes can be co-immobilized on one sponge, including phosphotriesterases (paraoxonase or OP hydrolases) and/or cholinesterases. The advantage of including OP hydrolases in the multi-enzyme component is that they detoxify all phosphonylated oximes with little substrate specificity.

4.4: In addition to decontamination of skin, wounds, and personnel, the enzyme-sponges can be utilized for preventing cross-contamination of medical and clinical personnel. Still more uses for these formulations could include decontamination foams as masks and in garments, replacing carbon filters that absorb OPs without inactivating them. The ChE-PUF could be used in chemical-biological sensors and incorporated into the telemedicine initiative as electrochemical OP probes. OPs in the environment could be contained and decontaminated if the ChE-PUF were incorporated into firefighting foams. The enzyme-foams could be used to decontaminate sensitive equipment without posing new environmental disposal problems, since the final products are rendered inert. Indeed, the sponge should be suitable for a variety of detoxification and decontamination schemes for both chemical weapons and civilians exposed to pesticides or highly toxic OPs such as sarin.

5. References

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